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(54) **Novel polypeptides, the DNA sequences allowing their expression, method of preparation, and utilization.**

(57) The present invention concerns a DNA sequence coding for a polypeptide with enantioselective amidase activity.

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NOVEL POLYPEPTIDES, THE DNA SEQUENCES ALLOWING THEIR EXPRESSION, METHOD OF PREPARATION, AND UTILIZATION

The present invention concerns polypeptides that possess an enantioselective amidase activity. It also concerns the genetic material required for the expression of these polypeptides as well as a microbiological procedure for their preparation. Finally, this invention concerns the utilization of these polypeptides and of transformed microorganisms for the enantioselective synthesis of acids from racemic amides, and in particular propionic acids, especially (S)-2-aryl-propionic acids and (R)-2-aryloxy-propionic acids.

Due to the presence of an asymmetric carbon atom, numerous molecules possess two distinct stereoisomeric forms, R and S, one being a mirror image of the other. This is the case for the 2-aryl-propionic acids. Most of the time, these molecules exist as a racemic mixture, with the two isomers present in more or less equal proportions. In certain cases, only one specific isomer is required, and it is therefore practical to have a means of separating the two isomers, or of performing a stereospecific synthesis of the desired isomer.

The present invention concerns the domain of polypeptides capable of hydrolyzing amides in an enantioselective manner : in particular, racemic 2-aryl-propionamides to (S)-2-aryl-propionic acids, and racemic 2-aryloxy-propionamides to (R)-2-aryloxy-propionic acids.

Among the microorganisms in which this enzymatic activity has been demonstrated, strains of the genus Brevibacterium and Corynebacterium stand out (European patent No. 89 400197.3), and in particular, Brevibacterium strain R312 (CBS 717.73). In addition, strains such as Rhodococcus possess this enzymatic activity.

The present invention involves the characterization and purification of these enantioselective amidase activities, as well as the cloning and sequencing of the genetic material responsible for their expression. In that which follows, the term "Amd" is used to designate all enantioselective amidase activities. The term "Amd sequence" designates all nucleotide sequences coding for said amidase activities.

In particular, the objective of the present invention is to obtain high levels of expression of these enantioselective amidases in different host organisms by using recombinant DNA techniques.

One of the goals of the invention therefore concerns the DNA sequences coding for these polypeptides with enantioselective amidase activity, especially with regard to racemic 2-aryl-propionamides. In a preferred embodiment of the invention, the object concerns the nucleotide sequence coding for the enantioselective amidase of Brevibacterium R312 (represented in Figure 8) or the enantioselective amidase of Rhodococcus (represented in Figure 13), as well as any degenerated sequences coding for the same polypeptide. The invention also concerns the sequences that hybridize with these DNA sequences or with fragments thereof and which code for polypeptides with enantioselective amidase activity. The invention also concerns the genes containing these DNA sequences.

Studies of the homology between the peptide sequences of these amidases reveal a highly conserved region responsible for the observed activity. This region corresponds to amino acids 137 to 193 of the peptide sequence shown in Figure 13 (nucleotides 618 to 788), and to amino acids 159 to 215 of the peptide sequence of the amidase of Brevibacterium R312 previously described, with which it shares a strict homology (67%).

One of the objects of the present invention therefore concerns a DNA sequence such as that described previously, characterized by the fact that it contains at least the sequence coding for amino acids 137 to 193 in Figure 13, or 159 to 215 in Figure 8, or a peptide sequence with at least 50% homology to these.

In particular, one of the objects of the present invention concerns a DNA sequence characterized in that it contains all or part of the Amd sequence presented in Figures 8 and 13, or a variant thereof. For the purposes of the present invention, "variant" is meant to describe all sequences that conserve the properties of the initial sequence, even if they contain alterations resulting from, for example, mutations, deletions, insertions, or degeneracy of the genetic code.

More precisely, the DNA sequence contains the sequence presented in Figures 8 or 13.

These sequences can be obtained by diverse methods. The general strategy is to clone the genomic DNA fragment coding for the desired polypeptide, with the aid of nucleotide probes derived from the purified polypeptide. By using different methods including primer elongation, restriction enzymes, insertion of adaptors, or ligation of linker oligonucleotides, a nucleotide insert containing the desired DNA sequence can be constructed. It can then be mapped and sequenced by techniques described in the literature.

Oth r techniques can be used as well, including the utilization of DNA and/or partial or total chemical synthesis. These techniques are well known, and the structures described in Figures 8 and 13 all w the isolation of an quivalent sequence, in any microorganism, using classical techniques.

In effect, having demonstrated the homology between the different enantioselective amidases, the present invention allows for the production of probes that can serve to identify hybridizing genes (i.e., genes with a sufficient homology) in any genomic bank. It is then easy to verify that such genes code for an enantioselective

amidase. In this manner, it is possible to obtain high quantities of amidase in any microorganism. It is also possible that novel enantioselective amidase activities will be revealed.

The present invention also concerns the polypeptides possessing an enantioselective amidase activity, that contain at least one of the following peptid sequences :

- sequences corresponding to amino acids 137 to 193 in Figure 13
- sequences corresponding to amino acids 159 to 215 in Figure 8
- sequences sharing at least 50% homology with these sequences.

Another object of the invention concerns novel polypeptides whose structure is derived from the DNA sequences previously described, and which possess an enantioselective amidase activity. These polypeptides are obtained by extraction and purification from cultures of natural or recombinant microorganisms. The purification is carried out in a succession of steps consisting of the preparation of crude extract from the culture, ammonium sulfate fractionation of the extract, and purification by chromatography and gel filtration. Details are given in the examples.

More precisely, the invention concerns the enantioselective amidases of Brevibacterium R312 and Rhodococcus.

The invention also concerns transformed microorganisms containing at least one expression cassette for the DNA sequences mentioned above. These cassettes will preferably be comprised of a DNA sequence according to the present invention, placed under the control of regulatory DNA sequences that insure its expression in the desired host. The cassette can be integrated in the host genome, or inserted in a plasmid carrying a selectable marker and an origin of replication functional in the host.

One of the interests of the present invention is the expression of these polypeptides under artificial conditions, i.e. the expression of a heterologous sequence in a certain cell whose culture conditions are particularly advantageous, and/or the expression of a homologous sequence under the control of at least partially heterologous regulatory signals in order to increase the production and/or ameliorate the culture conditions.

The DNA sequences controlling the expression of the DNA sequences that are the object of the present invention preferable carry a transcription and translation initiation region. This region contains a promoter and a ribosome binding site that can be homologous or heterologous to that of the peptide product.

The choice of regulatory region depends on the host to be used. In particular, for prokaryotic hosts, the heterologous promoter can be chosen from among the strong bacterial promoters, such as the promoters of the tryptophan operon Ptrp, the lactose operon Plac, the right or left promoters of bacteriophage lambda P_R and P_L, the strong promoters of corynebacteria phages, or even homologous promoters of corynebacteria. More precisely, in the case of the right promoter of lambda, the temperature sensitive form $P_R$clts is preferable. For eukaryotic organisms such as yeast, the promoters of the yeast glycolytic genes can be used, such as the promoters of the genes phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GPD), lactase (LAC4) and enolase (ENO).

When the host microorganism is prokaryotic, the sites of ribosome fixation will preferentially be derived from either the cII gene of lambda or from homologous genes of corynebacteria.

A transcription and translation termination region functional in the host will be placed 3' to the coding sequence. The plasmid will also carry one or several markers permitting a selection of the recombinant host. Dominant markers are preferred, such as those conferring resistance to antibiotics like ampicillin or streptomycin, or to other toxins.

The host microorganisms to be used notably include enterobacteria such as E. coli, and corynebacteria of the genus Corynebacterium, Brevibacterium, or Rhodococcus.

Of course, other cell types can be used, based on the same principle.

One object of the invention concerns the plasmids previously described containing at the least a transcription and translation initiation region, a DNA sequence coding for the desired polypeptide, and a selectable marker.

The invention also concerns the transformed microorganisms previously described, regarding their application in the preparation of enantioselective amidases as well as their use for enantioselective synthesis of acids from racemic amides.

The procedure for preparation of enantioselective amidases involves cultivation of the previously described microorganisms under conditions allowing expression of the sequence coding for the enantioselective amidase, followed by separation of the microorganisms from the amidase that has been produced.

More precisely, the invention concerns the utilization of the recombinant microorganisms or polypeptides already described, for the enantioselective synthesis of 2-aryl-propionic acids from the corresponding racemic 2-aryl-propionamides.

According to one of the preferred embodiments of the present invention, a recommended procedure is described that consists of the preparation of a stereoisomer of an organic acid from the corresponding racemic

amid , characterized in that the racemic amide is placed in the presence of the microorganism transformed as previously described, or in the presence of a polypeptide obtained as previously described, and the resulting stereoisomer is recovered.

Among th amides that can be subjected to this process, the racemic amide of ketoprof n should be mentioned, from which S(+) ketoprofen -useful in the pharmaceutical industry - can be repared.

The examples and figures that follow present other characteristics and advantages of the present invention. These should be considered as illustrative and non-limiting.

DESCRIPTION OF FIGURES

— **Figure 1** :

A. Peptide sequences (N-terminal and internal) obtained from the purified amidase from Brevibacterium R312.
B. Oligonucleotide probe derived from the internal peptide fragment.

— **Figure 2** :

A. Strategy for the design of probe Sq 918, from the N-terminal peptide fragment derived from the amidase of Brevibacterium R312.
B. Specific probe Sq 918.

— **Figure 3** :

A. Hybridization profile of probe Sq 918 with total genomic DNA from Brevbacterium R312 digested with EcoRI, HindIII, KpnI, PstI SmaI and SphI.
B. Hybridization profile of probe Sq 762 with total genomic DNA from Brevibacterium R312 digested with BamHI, BglII, EcoRI, KpnI, PstI, SalI, SmaI, SphI, SstI, and XhoI.

— **Figure 4** :

Restriction maps of plasmids pXL1650 and pXL1651.

— **Figure 5** :

Restriction map of the 5.4 kb PstI fragment containing the enantioselective amidase gene of Brevibacterium R312.

— **Figure 6** :

Sequencing strategy of the BamHI - PstI fragment containing the enantioselective amidase gene of Brevibacterium R312.

— **Figure 7** :

Analysis of the open reading frames of the sequenced fragment.

— **Figure 8** :

Nucleotide and peptide sequences of the enantioselective amidase gene of Brevibacterium R312.

— **Figure 9** :

Restriction map of plasmid pXL1724.

— **Figure 10** :

Restriction map of plasmid pXL1751.

— Figure 11 :

Restriction map of plasmid pXL1752.

— Figure 12 :

12.5% SDS-polyacrylamide gel after Coomassie blue staining, showing the expression of the enantioselective amidase of Brevibacterium R312 in strains E. coli B and E. coli K12 E103S. Each lane corresponds to a quantity of protein equivalent to 60 µl of the culture at an O.D. of 2.1 (E103S) or 0.7 (E. coli B). T, sonicated (pXL1029 and pXL906) contain the IL1-β gene under control of the $P_R$clts or Ptrp promoter, respectively.

— Figure 13 :

Nucleotide and peptide sequences of the enantioselective amidase gene of Rhodococcus (BamHI fragment from plasmid pXL1836).

— Figures 14 :

Restriction map of shuttle vector pSV73.

— Figures 15 :

Restriction map of expression plasmid pYG811B.

— Figures 16 :

Restriction map of expression plasmid pYG817B.

— Figures 17 :

Restriction map of expression plasmid pYG822.

STARTING PLASMIDS

Plasmid pXL1029 has been described in Jung et al. (1988), Ann. Inst. Pasteur/Microbiol. 139,129-146). It carries an EcoRI- NdeI fragment containing $P_R$clts-RBSclIΔtRI.

EXAMPLE 1

Identification and purification of the enantioselective amidase of Brevibacterium R312.

1.1. Identification

(R,S)-2-(4-hydroxy-phenoxy)-propionamide (HPP Amide), a derivative of 2-aryloxy-propionamide, is a better substrate for the enantioselective amidase than 2-aryl-propionamide derivatives, notably 2-phenyl-propionamide and 2-(3-benzoyl-phenyl)-propionamide. Furthermore, the selectivity of the amidase vis-à-vis the R enantiomer of HPP Amide is representative of the selectivity vis-à-vis the S enantiomer of 2-aryl-propionamide derivatives.

As a consequence, the enantioselective enzymatic activity was detected using 2-(4-hydroxy-phenoxy)-propionamide as substrate. The reaction was carried out at 25°C with agitation in a buffer of 50 mM sodium phosphate, pH 7.0, in the presence of Brevibacterium R312 ; it was stopped by addition of a mixture of 0.05 M phosphoric acid, acetonitrile, and 1 N HCl in a ratio of 55/40/5 (v/v). After centrifugation of the culture the supernatant was analyzed by reverse phas high performance liquid chromatography (HPLC) (Hibar-Merck RP-18,5 µm). Elution was performed with a solution of 0.005 M phosphoric acid and acetonitrile (85/15) (v/v). The respective concentrations of HPP Amide and HPP Acid were measured by comparing the elution peaks to a standard. For this substrate, the nanti meric excess is defined as (R - S)/(R + S) x 100 wh re R and S are the respective concentrations of the R and S nantiomers of HPP Acid. Th nantiomeric excess was deduced

eith r from polarimetric measurement (using the absorption of sodium at 589 nm), or by HPLC using a chiral column.

Th activities obtained with whole cells and a soluble extract, respectively, were 15 U/mg and 24 U/mg of protein, (1 U = 1 µm l HPP Acid f rmed per hour). The enanti meric excess of (R)-HPP Acid is 95 %. These results demonstrate that Brevibacterium R312 possesses an enantioselective amidase capable of hydrolyzing racemic 2-aryl-propionamides to the corresponding S acids. This was verified by the hydrolyses of racemic 2-phenyl-propionamide and racemic 2-(3-benzoyl-phenyl)-propionamide to the respective corresponding S acids, with an enantiomeric excess higher than 93%.

1.2 Purification

The purification was carried out at 4°C. Cells (40 g dry weight Brevibacterium R312) were thawed and suspended in 300 ml Buffer A (50 mM sodium phosphate, pH 7,5 mM β-mercaptoethanol). Cells were then broken by sonication and membrane debris were eliminated by centrifugation at 20000 rpm for 30 minutes. To 30 ml of supernatant, 25 ml of a 10% solution of streptomycin sulfate was added slowly, with stirring. After 45 minutes, the solution was clarified as above and the resulting supernatant was treated with ammonium sulfate. The protein fraction precipitating between 30.8% and 56.6% saturation of ammonium sulfate was collected by centrifugation and dissolved in 35 ml Buffer A, and then dialyzed slowly against the same buffer. The solution thus obtained was adjusted to 20% saturation of ammonium sulfate, centrifuged, then applied to a phenyl-Sepharose CL-4B column (Pharmacia) equilibrated with Buffer A at 20% saturation of ammonium sulfate. Active fractions were eluted with the same buffer, then concentrated by ultrafiltration to a volume of 18 ml using an Amicon Diaflo PM10 cell. Glycerol (10%) was then added to the concentrated solution, and the resulting solution was applied to an Ultrogel AcA 44 column (IBF-Biotechnics, France) previously equilibrated with 50 mM Tris-HCl, pH 7, 100 mM NaCl. Fractions containing the highest specific activity (approximately 32% of the total activity loaded onto the column) were collected, concentrated, and subjected to a supplementary filtration step on the same gel. In parallel, fractions containing the highest specific activity (approximately 30% of the total protein loaded onto the column) were analyzed by SDS-PAGE and stored. The enantioselectivity of the purified protein was also determined.

This purification method resulted in an enzyme more than 80% pure, with a specific activity of 815 U/mg. At this step, a major band of apparent molecular weight 59 +/- 5 KD which corresponds to at least 80% of the total proteins, is visible on SDS-PAGE. Moreover, the amidase activity eluted from an HPLC TSK 3000 column corresponds to a molecular weight of 122 KD, indicating that the enzyme is in a dimeric form.

Table 1 shows the characteristics of the different fractions. This table describes the different steps of the purification of the enantioselective amidase of Brevibacterium R312 :

- from 40 g of humid cells, after precipitation with streptomycin sulfate
- one unit (U) corresponds to 1 µmol HPP Acid formed per hour under the conditions described below.

Table 1

Purification Step	Vol. (ml)	Quantity of protein (mg)	Activity (U/mg)	Yield %	Purification Factor
1/ Crude extract	325	1918	26.4	100	1
2/ Ammonium sulfate precipitate	29.5	613	62.5	75	2.4
3/ Phenyl-sepharose eluate	77	200	198	78	7.5
4/ AcA44, first eluate	6	27	457	24.4	17.3
5/ AcA44, second eluate	3	3.9	815	6.3	31

EXAMPLE 2

Cloning the enantioselective amidase of Brevibacterium R312

2.1 Derivation of protein sequences

The peptide sequences corresponding respectively to the N-terminal extremity (27 residues) and a trypsic internal fragment (21 residues) of the enantioselective amidase of Brevibacterium R312 were determined using the purified enzyme.

This was done by subjecting 3 nmol of the amidase preparation to reduction and carboxymethylation. The major protein component was then desalted, and purified to homogeneity by reverse phase HPLC. The N-terminal sequence was then determined by the Edman method of automatic sequential degradation, using an Applied Biosystems Model 470A instrument. The sequence presented in Figure 1A was obtained in this manner. To obtain the additional internal sequence, the same quantity of protein was digested with trypsin. The reduced and carboxymethylated fragments were then separated by reverse phase HPLC (2.1 × 10 mm, flow 0.2 ml/min) using the following elution buffer : a gradient of 0 to 50% acetonitrile in 0.07% trifluoroacetic acid. The peptide eluting in a well-separated peak (at 40.8% acetonitrile) was sequenced (Figure 1A).

2.2 Construction of the nucleotide probes

Two strategies w re pursued.

In th first strategy, a 29-mer probe (59% minimal homology) was constructed, ke ping in mind the cod n usage in the tryptophan operon of Brevibacterium lactof rmentum (7.7 kb sequence containing 6 cistrons : Matsui et. al., Mol. Gen. Genet. 209 p. 299, 1987), and using the sequence IDGALGSYDV of th internal fragment (presenting a smaller average degeneracy). The noncoding strand was synthesized with consideration of the relative thermodynamic neutrality of G : T pairing and by introducing several degeneracies in ord r to maximize

the averag theoretical frequency of codons considered (88% in relation to th usage of the chosen cod ns). These consid rations led to a GC content in the probe of about 69%. The sequence of the probe (Sq 762) is shown in Figure 1B.

In the second strategy, the PCR method described by Girgès et. al. (Nucleic Acids Res. 16, p. 10371, 1988) was used to obtain an exact nucleotide probe from a peptide corresponding to highly degenerated codons. To accomplish this, 25-mer oligonucleotides (see underlined sequences in Figure 2A) were synthesized, corresponding to all the possibilities of coding of the first or last five codons of the N-terminal peptide sequence, and carrying EcoRI and HindIII sites respectively, at their 5' extremities. These 25-mers were used to prime an enzymatic amplification of Brevibacterium R312 genomic DNA. After 30 cycles of amplification the candidate fragment was purified on a gel, then inserted between the HindIII and EcoRI sites of bacteriophage M13mp19. In fact, two different hybridization temperatures of the primer (45°C and 48°C) were used, resulting in two candidate fragments. Thus after cloning the fragments, several clones from each temperature were sequenced and compared. The results are shown in Figure 2A. It can be seen that apart from the degeneracies introduced by the primers, a DNA fragment (unique between primers) coding for the N-terminal extremity of amidase was well amplified. A 40-mer synthetic oligonucleotide (Sq 918) corresponding to this internal fragment was therefore used for the rest of the clonage as an exact probe for the N-terminal extremity of amidase. Figure 2B shows the nucleotide sequence of specific probe Sq 918.

The two probes Sq 762 and Sq 918 thereby obtained were labeled by 5'phosphorylation with ^{32}P.

2.3 Cloning of the gene encoding the enantioselective amidase of Brevibacterium R312

The strategy consisted of first verifying the specificity of the probes and determining the nature of the genomic DNA fragment to be cloned by Southern blot. Briefly, Brevibacterium R312 genomic DNA was alternatively digested by several restriction enzymes corresponding to possible cloning sites, and in particular to sites present in the multisite cloning region of pUC plasmids. Notably, PstI was used. After electrophoresis through an agarose gel and transfer to a nylon membrane, the various digestions were hybridized to probes Sq 762 and Sq 918. The results shown in Figure 3 demonstrate that the two probes present a sufficient specificity under the conditions of hybridization (at most one fragment hybridizing for each digestion). Furthermore, since the two probes give almost the same profile of hybridization, one might be led to believe that the hybridization signals of the sought-after gene are rather specific, and that the internal peptide obtained after trypsic digestion is very close to the N-terminal extremity. In particular, the hybridization footprints reveal the existence of a unique 5.4 kb PstI fragment that hybridized strongly with the two probes. It was therefore decided to clone this fragment.

For the cloning, all fragments of approximate size between 4.6 and 5.5 kb and 5.5 to 6.5 kb resulting from the PstI digestion of total genomic Brevibacterium R312 DNA, were purified on agarose, electroeluted, and ligated to pUC19 cut with PstI. After transformation of E. coli strain DH5α, 500 white colonies were obtained on X-gal medium, which theoretically correspond to recombinant microorganisms. Each colony was individually isolated, transfered onto a nylon membrane, then analyzed by hybridization with the ^{32}P-labeled Sq 918 probe. Two clones hybridized very strongly ; they were isolated and used in following steps.

The two recombinant plasmids pXL1650 and pXL1651 isolated from these two clones were analyzed by various methods, including restriction mapping, partial sequencing using the probes as sequencing primers, and Southern blot. Figure 4 shows that the two plasmids contain the same 5.4 kb PstI insert, in the two orientations. Figure 5 shows the restriction map of this fragment. These two plasmids indeed contain the sequences coding for the characterized peptides, the tryptic fragment adjoining the N-terminal (Figure 8). Furthermore, these results show that the gene coding for the enantioselective amidase of Brevibacterium R312 is located on a 2.3 kb BamHI-PstI fragment, oriented in the sense BamHI toward PstI. Given the position of the 5' extremity of the coding sequence and knowing that the enzyme is coded by at most 2 kb (57 - 63 KD monomer according to our estimations), it is certain that the entire gene was contained in the BamHI- PstI fragment that we therefore proceeded to sequence.

EXAMPLE 3

Sequenc of the BamHI-PstI fragment containing the gene encoding the nantioselective amidas of Brevibact rium R312

Th sequencing strategy for the BamHI-PstI fragment is shown in Figure 6. The various s quences were all obtained by the chain termination meth d (Sequenase kit in the pres nce of 7-deaza-dGTP ; (^{35}S)-dATP) ither on single stranded M13 matrices carrying subfragm nts, or directly on plasmid pXL1650. To this end,

several specific primers were also synthesized. The average GC content of the sequence obtained is 61.5%. Figure 7 presents an analysis of the open reading frames ; it is seen that the open reading frame corresponding to the amidase codes for 521 amino acids, a protein of calculated molecular weight of 54671. The GC content of this open reading frame is respectively 65.8%, 52.5% and 70% for the first, second and third codon positions, which is a typical distribution in coding sequences of GC-rich microorganisms. Figure 8 shows the complete sequence of the BamHI-PstI fragment.

EXAMPLE 4

Expression in E. coli of the gene encoding the enantioselective amidase of Brevibacterium R312

4.1 Construction of plasmids

Several plasmids were constructed in which the structural gene of amidase, containing a homologous ribosome binding site (RBS) or the RBS from the cII gene of lambda, was placed under the control of its own promoter, the promoter of the tryptophan operon, or the right temperature sensitive promoter of lambda. Plasmid pXL1650 (Figure 4) was obtained by insertion of the 5.4 kb fragment resulting from the PstI digestion of total Brevibacterium R312 genomic DNA, into the unique PstI site of plasmid pUC19. This plasmid therefore carries the promoter of the lactose operon Plac, followed by a ribosome binding site and the structural gene encoding the enantioselective amidase of Brevibacterium R312, as well as a marker encoding ampicillin resistance.

Plasmid pXL1724 (Figure 9) contains the 2.3 kb BamHI-PstI fragment excised from the 5.4 kb PstI fragment under control of the promoter of the tryptophan operon of E. coli. In this construct, the amidase gene of Brevibacterium R312 is therefore preceded by 58 base pairs upstream of the ATG codon containing its own ribosome binding site (Figure 8).

Two other constructions were made in which the structural gene encoding the enantioselective amidase of Brevibacterium R312 was placed under the control of heterologous promoters, with heterologous ribosome binding sites. These plasmids (pXL1751 and pXL1752) were obtained as follows :

Plasmid pXL1724 was mutagenized by PCR in order to substitute an NdeI site (CATATG) for the ATG codon situated upstream of the amidase structural gene. Amplification was carried out using a primer corresponding to the NdeI site hybridizing with the initiation ATG codon, and a primer corresponding to an XhoI site situated downstream of the ATG codon. The amplified fragment was then excised by digestion with NdeI and XhoI.

- Utilization of promoter Ptrp :

Into plasmid pXL1724 digested by EcoRI and XhoI, was inserted an EcoRI-NdeI fragment carrying the Ptrp promoter and the ribosome binding site of the lambda cII gene in which the termination sequence tR_1 has been deleted, and the 5' region of the amidase structural gene (fragment NdeI-XhoI). This generated plasmid pXL1751 (Figure 10).

- Utilization of promoter $P_R$clts :

The same strategy was employed, this time by using the EcoRI NdeI fragment from plasmid pXL1029 containing the $P_R$clts promoter and the ribosome binding site of the lambda cII gene deleted of the termination sequence tR_1. This generated plasmid pXL1752 (Figure 11).

4.2 Expression of the amidase gene of Brevibacterium R312 in E. coli B and E. coli K12 E103S

Plasmids pXL1751 and pXL1752 were used to transform strains E.coli B, and E. coli K12 E103S, respectively, by the calcium chloride method. Selection of recombinant microorganisms was carried out in ampicillin medium.

The expression of the enantioselective amidase of Brevibacterium R312 was measured after sonication of the cells, by SDS-PAGE of the crude fractions or, after centrifugation, of the pellet and supernatant. The results in Figure 12 show a high level of amidase expression, representing up to 20% of total protein.

EXAMPLE 5

Utilization of the enantioselective amidase of Brevibacterium R312 for the enantioselective synthesis of 2-aryl-propionic acids

The following strains were used in that which follows :

E. coli (pXL1751) - the amidase coding sequence is placed under the control of the promoter of the tryptophan

op ron.

E. coli (pXL1752) - amidase is produced by raising the temperature from 30°C to 42°C at the end of th xpo-n ntial phase (P_R promot r of lambda under control of the temperature sensitive repressor clts).

Tw control strains were also used :

E. coli (pXL906) - equivalent to E. coli (pXL1751) with the amidase gene replaced by the gene IL1β.

E. coli (pXL1029) - equivalent to E. coli (pXL1752) with the amidase gene replaced by the gene IL1β.

The following procedure was used to test the activity of these microorganisms :

A cell suspension grown under appropriate inducing conditions was added to a solution containing :

- hydroxy-4-phenoxy-2-propionamide (HPPAm), or
- phenyl-2-propionamide (PPAm), or
- the amide of ketoprofen (KAm), for example.

The reaction mixture was then diluted in a buffer containing acetonitrile : N hydrochloric acid (90 :10) (v/v), and the cells were eliminated by centrifugation. The reaction mixture was resolved by HPLC and the amidase activity was calculated. The results shown in Table 2 demonstrate the efficiency of this system.

Table 2 shows the specific activity of the amidase of Brevibacterium R312, as produced in E. coli in inducing conditions, toward the racemic substrates HPPAm, PPAm and KAm, as well as the enantiomeric excess of the chiral acids produced. In this experiment, E. coli strains harboring plasmids pXL1751 (amidase) or pXL906 (control) were grown at 37°C.

Table 2

E. coli strains in inducing conditions	Specific activity µmol/h/g protein			Enantiomeric excess %		
	HPPAm	PPAm	KAm	HPPA R+	PPA S+	Keto S+
pXL 1751	1300	50	4	93	96	95
pXL 1752	1300	50	5	94	97	95
pXL 906	0	nd	nd	nd	nd	nd
pXL 1029	14	0	0	nd	nd	nd

Table 3 shows the specific activity of the amidase of Brevibacterium R312 (expression plasmid pXL1751), as produced in E. coli grown at 28°C in induced or repressed conditions, toward the racemic substrates KAm, as well as the enantiomeric excess of the chiral acid produced.

Table 3

Bacterial strain	Plasmid	Repressor (1)	Specific activity µmol/h/g protein	ee (%)
E. coli	pXL1751	-	55	96
"	"	Trp	13	nd

nd = not determined. ee : enantiomeric excess (%).

Note (1) = Trp : L-tryptophane.

Therefore, E. coli strains harboring the amidase gen of Brevibacterium R312 (g notype Amd+) can efficiently hydrolyze the following three amides (phenotype AMD+) :

- 2-(4-hydroxy-phenoxy)-propionamide (HPPAm)
- 2-phenyl-propionamid (PPAm)
- amide of ketoprofen (KAm).

The enantiomeric excess obtained was always greater than 93%.

EXAMPLE 6

Purification of the nantioselective amidas of Rhodococcus

I. Assay of enzymatic activity

The active fraction was incubated at 30°C for 30 minutes in 500 µl of buffer (0.1 M Tris HCl pH 7.5, 5 mM DTT, 18 mM 2-phenyl-propionamide). After incubation, 2 ml of a mixture of acetonitrile/HCl 1N (90/10) and then 2 ml of a mixture of 50 mM H_3PO_4/CH_3CN (75/25) were added to the reaction mixture. After centrifugation at 5000 rpm for 10 minutes, an aliquot of the supernatant was subjected to HPLC to measure the reaction products.

- — Column : Nucleosil 5-C18 25 cm
- — Eluant : 50 mM H_3PO_4/CH_3CN (75/25)
- — Loading : 10 µl
- — Flow rate : 1 ml/min

A unit of activity is defined as the quantity of enzyme necessary for he hydrolysis of 1 µmol 2-phenyl-propionamide per hour.

II. Purification protocol

6.1 Preparation of the enzyme extract

7 g of cells were suspended in 15 ml 0.1 M Tris HCl pH 7.5, 5 mM DTT, and sonicated for 15 minutes at 4°C. The crude enzyme extract was collected by centrifugation at 50000 rpm for 1 hour.

6.2 First ion-exchange chromatography

To 20 ml of crude extract, 20 ml of Buffer A (25 mM Tris HCl pH 7.5, 5 mM DTT) was added. The sample was injected onto a Mono Q HR 10/10 column (Pharmacia) equilibrated in Buffer A, at a flow rate of 3 ml/min. After washing the column, the proteins were eluted with a linear 1 hour gradient of 0.1 to 1 M KCl at a flow rate of 3 ml/min. Fraction size was 6 ml. The amidase eluted in 18 ml at approximately 0.3 M KCl.

6.3. Second ion-exchange chromatography

The active fractions were combined and concentrated to 2 ml using a Centriprep ultrafiltration system (Amicon). After dilution with 6 ml Buffer A, 4 ml of the sample was injected at 1 ml/min onto a Mono Q HR 5/5 column equilibrated in Buffer A. Proteins were eluted with a linear gradient of 0 to 0.5 M KCl in Buffer A. Active fractions were combined and adjusted to 15% glycerol (v/v), then concentrated to 1 ml as above.

6.4 Hydrophobic chromatography

1 ml of Buffer B (0.1 M Tris HCl pH 7.5, 0.5 mM DTT, 1.7 M $(NH_4)_2SO_4$) was added to the sample which was then injected (in two injections) onto a Phenyl-Superose HR 5/5 column (Pharmacia) at a flow rate of 0.25 ml/min. Proteins were eluted at 0.5 ml/min with a decreasing linear gradient of $(NH_4)_2SO_4$ (1.7 M to 0 M) in 25 ml. Fraction size was 0.5 ml. Active fractions were adjusted to 15 % glycerol then diluted to 1 ml with Buffer A.

6.5 Hydroxyapatite chromatography

The sample was injected at 0.5 ml/min onto a Bio-Gel HPHT column (Bio-Rad) equilibrated with Buffer C (85 mM Tris HCl pH 7.5, 0.5 mM DTT, 10 µM $CaCl_2$, 15% glycerol). The amidase was eluted at a flow rate of 0.5 ml/min with a linear gradient of 0 to 100% of buffer 0.35 M potassium phosphate pH 7.5, 0.5 mM DTT, 10 µM $CaCl_2$, 15% glycerol in Buffer C, in 20 minutes.

These steps allow the purification to homogeneity of an enzyme with a specific activity of 988 U/mg of protein.

The nzym thereby btained is present in th form of a dim r of identical subunits of apparent molecular weight 53 +/- 2 KD.

EXAMPLE 7

Cloning f th gene encoding this amidase

After a suppl mentary purification step on TSK-G3000 SW, the enzyme was subjected to s quencing. The N-terminal extremity was inaccessible to Edman-type chemistry, and so a total trypsin hydrolysis was carri d out and three HPLC fractions of the hydrolysate—123, 124 and 162—provided peptides that allowed an unambiguous sequence to be obtained. From the sequence obtained from fraction 123, a 32-mer nucleotide probe was synthesized, corresponding to a mixture of 8 oligonucleotides and containing 7 inosines in positions degenerated at least three times :

Probe A (from peptide 123)

A T V D V P V P D Y A

5' 3'

GCIACIGTIGATGTICCIGTICCIGATTATGC

C C C

The efficiency of this probe, labeled at the 5' end with ^{32}P, was tested by Southern transfer onto genomic DNA from Rhodococcus previously digested by one of the following restriction enzymes : SstI, SphI, SmaI, PstI, KpnI, EcoRI, SalI and BamHI. Experimental conditions were as follows : hybridization buffer, 5x SSC, 5x Denhardt, 0.1% SDS, 50 mM $NaPO_4$ pH 6.5, 250 µg/ml salmon sperm DNA ; hybridization temperatures were 50°C or 55°C (two experiments) ; wash conditions were 1 hour in 6x SSC at room temperature and 5 min. in 2x SSC, 0.1% SDS at 50°C.

Under these conditions, probe A gave strong, unambiguous signals ; in particular, with the BamHI, KpnI, SphI, SstI, SmaI, SalI, and PstI digestions, a single genomic band was found, strongly hybridizing to probe A. For PstI digestion, the size of the hybridizing signal to probe A corresponds to a genomic fragment of approximately 3.2 kb.

The 3 to 4 kb PstI digestion fragments of genomic DNA were thus purified by preparative electrophoresis through agarose followed by electroelution, then ligated to plasmid pUC19 that had been cut by PstI. After transformation of E. coli strain DH5α, 600 clones that were white on LB Amp-X-gal were repicked individually and probed with probe A by colony hybridization, in stringency conditions similar to the Southern. The 9 clones with particularly strong hybridization signals were then analyzed by restriction of plasmid DNA. Among 6 of these clones having clearly inserted the same 3.2 kb fragment in the two orientations, 2 clones representing each orientation (pXL1835 and pXL1836) were analyzed in more detail (detailed mapping, Southern analysis), thereby confirming that the desired fragment had been obtained.

EXAMPLE 8

Sequence of the 3,2 kb PstI fragment

The complete nucleotide sequence of the 3.2 kb PstI fragment was determined for the two strands. The GC content of this fragment was 62.4 %, similar to the GC content of R312 (approximately 62%). Analysis of the sequence revealed an open reading frame of 1386 nucleotides (position 210 to 1595) coding for a polypeptide of 462 amino acids (calculated molecular weight of 48554) that contained the three peptide previously obtained by sequencing the trypsic fragments. This open reading frame is included in a BamHI subcloned fragment whose nucleotide sequence is shown in Figure 13.

The 3 underlined peptide sequences correspond to the peptide fragments determined directly on the trypsic fragments of the purified enzyme (peptide 123, 124 and 162). The underlined nucleotide sequence corresponds to the (degenerated) probe used to clone the gene. The peptide sequence in italics corresponds to residues 137 to 193 that are highly conserved between the enantioselective amidases of Brevibacterium strain R312 and the strain of the genus Rhodococcus (see below).

This open reading frame represents the structural gene of the nantioselective amidase.

EXAMPLE 9

Homol gies betwe n different amidases : identification of a sequence characteristic of amidase activity

A comparison of the peptide sequences of the nantioselective amidase of R312 (Figure 8) and th amidase shown in Figure 13 shows a strong homology in about two-thirds of the sequence, between residues 150 and 300 of R312 (50% strict id ntity), with the homology reaching 67 % between residues 159 and 215.

A search of the GENPRO gene bank for homologous sequences reveal d some strong homologies between the 150 to 200 region, and the sequences of the acetamidase of Aspergillus nidulans, the indolacetamide hydrolases (IAH) of Pseudomonas syringae and Bradyrhizobium japonicum, the tms2 protein of Agrobacterium tumefaciens, and the 6-aminohexanoate-cyclic-dimerhydrolyases (ACDH) of Flavobacterium strain K172 and Pseudomonas strain NK87.

Table 4 shows the homology of peptide 137-193 of the amidase described above, with the respective sites of these other enzymes (expressed as % strict identity of amino acids) :

Table 4

Amidase	% homology
R312	65.5
tms2 A. tumefaciens	64.3
IAH P. syringae	61.8
ACDH (F. K172 or P. NK87)	61.4
IAH B. japonicum	54.4
Acetamidase (A. nidulans)	47.4

This strongly conserved region is most likely responsible for the activity of these enzymes (catalytic site).

EXAMPLE 10

Expression of the enantioselective amidase in E. coli

In order to confirm the identification of the phase coding for the enantioselective amidase, an NdeI site (CATATG) was created by PCR at the presumed ATG codon at position 210 (Figure 13), and the fragment between this site and the SalI site at position 1683, containing uniquely the region coding for amidase, was placed under the control of signals functional in E. coli for transcription initiation (promoters Ptrp or P_R) and translation (ribosome binding site cII). The vectors thereby obtained (pXL1893, Ptrp ; and pXL1894, P_R-cIts) are similar to vectors pXL1752 and pXL1751 expressing the amidase of R312, as previously described. Expression from plasmids pXL1893 and pXL1894 was studied in E. coli B and E. coli K12 E103S, respectively. A protein comigrating with the purified amidase was produced specifically at 42°C in the presence of plasmid pXL1894.

EXAMPLE 11

Expression of the enantioselective amidase in corynebacteria

1. Construction of the expression vectors

These vectors are derived from replicating vectors for corynebacteria. They include
- a replicon of E. coli
- a replicon of corynebacteria
- a selectable marker
- an Amd sequence.

Vector pSV73 (Figure 14) : this plasmid is derived from plasmid pSR1 of C. glutamicum (Yoshihama et. al., J. Bacteriol. 162, 591, 1985) by insertion of plasmid pUC8 containing an E. coli replicon and the kanamycin resistance gen carried on transposon Tn903.

This plasmid was used to construct th different expression vectors for the Amd sequences shown in Figure 13, n tably :

- V ctors pYG811A and B (Figure 15). These expression vectors are btained by cloning the Amd sequence contained in th SalI fragment represented in Figure 13 into the SalI site of pSV73, in both orientations.
- Vectors pYG817A and B (Figure 16). These expression vectors are obtained by cloning the Amd sequence contained in the BamHI fragment represented in Figure 13, into the BglII, site of pSV73, in both orientations.
- Vector pYG822 (Figure 17). This expression vector is derived from pSV73 by inserting between the SalI and BglII sites an expression cassette containing the Amd sequence shown in Figure 13 under control of the Ptrp promoter of the tryptophan operon of E. coli.
- Other cryptic corynebacterium plasmids can be used for the construction of expression vectors for the Amd sequence that are functional in corynebacteria. For example, plasmid pX18, isolated from B. lactofermentum (Yeh et. al., Gene, 47, 301-306, 1986), allowed the construction of shuttle vectors pYG820A and pYG820B which can replicate in Brevibacterium R312 and therefore can be used as recipients for cloning and expression experiments in several corynebacteria.

2. Transformation of corynebacteria

All known transformation techniques can be used, and notably the protoplast - regeneration technique described by Yoshima et. al. cited above. However the applicants have shown that the electroporation technique is very efficient, augmenting the frequency of transformation up to 1000-fold.

SDS-PAGE analysis of sonicated cells is used to investigate the intracellular expression of the enzyme in the recombinant hosts.

EXAMPLE 12

Enzymatic catalysis

This example illustrates the usage of Amd-type proteins, or the recombinant microorganisms expressing these proteins, for the enantioselective synthesis of optically active organic acids by hydrolysis of the corresponding racemic amides.

1. Preparation of the cells

The different strains were cultured in 2 liter erlenmeyer flasks in 600 ml medium, at 28°C in appropriate culture conditions with an agitation of 150 turns/min. After termination of the culture, cells were harvested, washed in a solution of NaCl (9 g/l) and stored at -18°C.

2.2-phenyl-propionamide as substrate

The protocol is as follows :

The 2-phenyl-propionamide and the cell suspension were added to a flask equipped with a stirrer, and the volume was adjusted to 5 ml with 50 mM potassium phosphate buffer pH 7.0. The flask was placed in a thermostated crystallizing dish at 25°C with stirring for 1 hour. The reaction mixture was then diluted with a solution of acetonitrile/HCl (9/1), (v/v), and bacteria and cell debris were eliminated by centrifugation. The composition in acid and amide was determined by HPLC.

The results obtained in Brevibacterium R312 and Brevibacterium lactofermentum (ATCC 21086) are as follows :

Table 5

Strain	Plasmid	Specific activity µmol/h/mg protein
Brevibacterium R312	pSV73	0.1
" "	pYG811A	4.3
" "	pYG811B	5.4
B. lactofermentum	pSV73	0
" "	pYG822	2.8

3. Racemic ketoprofen amide as substrate

As shown in Table 6, it is seen that recombinant corynebacteria expressing the amidase from Rhodococcus gave significantly higher activities than from control cells transformed with pSV73.

Table 6

Bacterial strain	Plasmid	Inducer (1)	Specific activity µmol/h/mg protein	ee (%)
Brevibact. R312	pSV73	IBN	0.01	nd
" "	pYG811A	IBN	0.04	96
" "	pYG811B	IBN	0.04	94
B. lactofermentum	pSV73	IBN + IBNAm	0	nd
" "	pYG822	IBN + IBNAm	0.02	nd

nd = not determined. ee : enantiomeric excess (S+ ketoprofen).
Note (1) = IBN : isobutyronitrile ; IBNAm : isobutyramide.

Claims

1. A DNA sequence coding for a polypeptide with enantioselective amidase activity.

2. A DNA sequence according to claim 1, characterized by the fact that it is chosen from :
   - the sequence coding for th enantioselective amidase of Brevibacterium R312 shown in Figure 8 and th sequ nce coding for the enantioselective amidase of Rh dococcus shown in Figure 13
   - an analog of these sequences coding for the same protein but resulting from the degeneracy of the genetic code
   - DNA hybridizing with one of these sequences or with a fragment thereof and coding for a polypeptide with nantioselective amidase activity

3. A DNA sequence according to claim 1, characterized by the fact that it includes at least the sequence coding for amino acids 137 to 193 shown in Figure 13, or amino acids 159 to 215 shown in Figure 8, or a peptide sequence with at least 50% homology to these sequences.

4. DNA which contains at least the coding region of the sequence shown in Figure 8.

5. DNA which contains at least the coding region of the sequence shown in Figure 13.

6. The gene containing the DNA sequences according to one of the claims 1 through 5.

7. Novel polypeptides resulting from the expression of a DNA sequence according to one of the claims 1 through 6, and possessing an enantioselective amidase activity.

8. Polypeptide according to claim 7, characterized by the sequence shown in Figures 8 or 13.

9. Polypeptide possessing an enantioselective amidase activity and presenting at least a sequence chosen among :
   - sequences corresponding to amino acids 137 to 193 of Figure 13
   - sequences corresponding to amino acids 159 to 215 of Figure 8
   - sequences sharing at least 50% homology with the preceding sequences.

10. Polypeptide according to one of the claims 7 through 9, characterized by the fact that it is not of natural origin.

11. Transformed microorganism containing at least an expression cassette carrying a sequence according to one of the claims 1 through 6 under the control of DNA sequences assuring the expression of this sequence in the host organism.

12. Microorganism according to claim 11, characterized by the fact that the DNA sequences assuring the expression of a sequence according to one of the claims 1 through 6 contain a transcription and translation initiation region.

13. Microorganism according to claim 11, in which the transcription and translation initiation region contains a promoter sequence and a ribosome binding site.

14. Microorganism according to claim 13, in which the promoter sequence and the ribosome binding site can be homologous or heterologous regarding the peptide produced.

15. Microorganism according to claim 14, characterized by the fact that the promoter sequences can be chosen from among the strong promoters of corynebacterium phages, the Ptrp promoter of the tryptophan operon, the Plac promoter of the lac operon, the left promoter P_L of phage lambda, or the right promoter P_R of the phage lambda.

16. Microorganism according to claim 15, in which the promoter is chosen from among the Ptrp promoter of the tryptophan operon and the right promoter $P_R$clts of the phage lambda.

17. Microorganism according to claim 11, in which the ribosome binding site is derived from the cII gene of phage lambda, or from homologous genes of corynebacteria.

18. Microorganism according to one of the claims 11 through 17, characterized in that the expression cassette is carried on a plasmid that also carries a means of selection.

19. Microorganism according to claim 11, in which the means of selection is a selectable marker that confers resistance to an antibiotic.

20. Microorganism according to claim 11, characterized in that the plasmid contains the Ptrp promoter of the tryptophan operon, the ribosome binding site of the cII gene of phage lambda deleted of the transcription termination sequence tR1, the DNA coding for the enantioselective amidase gene of Brevibacterium R312,

and a gen conferring ampicillin resistance.

21. Microorganism according to claim 11, characterized in that the plasmid contains the temperature sensitive right promoter of phage lambda P_RcIts, the ribosome binding site of the cII gene of phage lambda deleted of the transcription termination sequence tR1, the DNA coding for the enantioselective amidase gene of Brevibacterium R312, and a gene conferring ampicillin resistance.

22. Microorganism according to one of the claims 11 through 21, characterized in that it is chosen from among the strains E. coli, Brevibacterium, Corynebacterium, Rhodococcus.

23. Microorganism according to claim 22, characterized by the fact that it is strain E. coli B or E. coli K12 E103S.

24. Procedure for the preparation of an enantioselective amidase, characterized in that the microorganism according to one of the claims 11 through 23 is cultivated under conditions that allow expression of the sequence coding for the amidase, and that after culture the microorganism is separated and the amidase is extracted.

25. Procedure according to claim 24, characterized in that the culture is sonicated, fractionated with ammonium sulfate, chromatographed on phenyl-Sepharose, and subjected to a double gel filtration.

26. Procedure for the preparation of a stereoisomer of an organic acid from the corresponding racemic amide, characterized in that the racemic amide is placed in the presence of a microorganism described in one of the claims 11 through 23 or in the presence of a polypeptide described in one of the claims 7 to 10, and that after the reaction the stereoisomer is recovered.

27. Procedure according to claim 26, characterized in that the amide is a racemic 2-aryl-propionamide and the acid is an (S) acid.

28. Procedure according to claim 27, characterized in that the racemic 2-aryl-propionamide is the amide of ketoprofen and the acid is S(+) ketoprofen.

29. Procedure according to claim 26, characterized in that the amide is a racemic 2-aryloxy-propionamide, and the and is the corresponding R stereoisomer.

A

N-TERMINAL FRAGMENT
Ala Thr Ile Arg Pro Asp Asp Lys Ala Ile Asp Ala Ala Ala
Arg His Tyr Gly Ile Thr Leu Asp Lys Thr Ala (Arg) Leu ...

INTERNAL FRAGMENT
Leu Glu Trp Pro Ala Leu Ile (Asp) Gly Ala Leu
Gly Ser Tyr Asp Val Val Asp Gln Leu Tyr ...

B

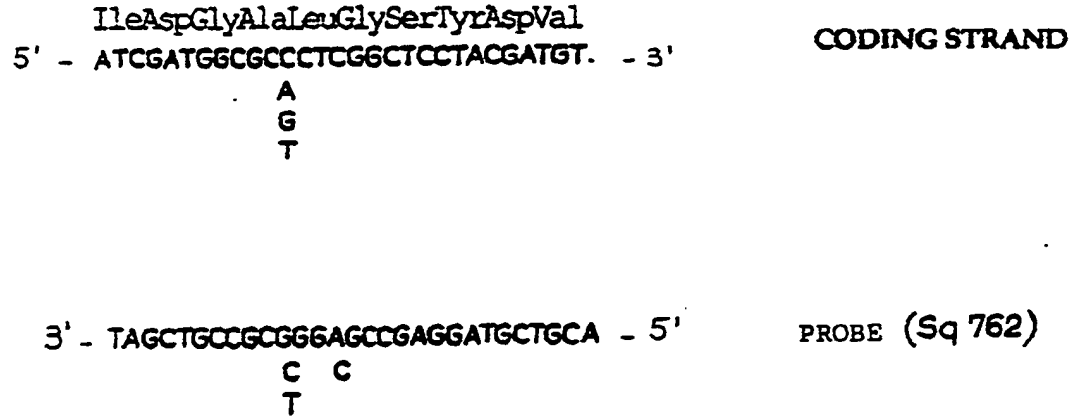
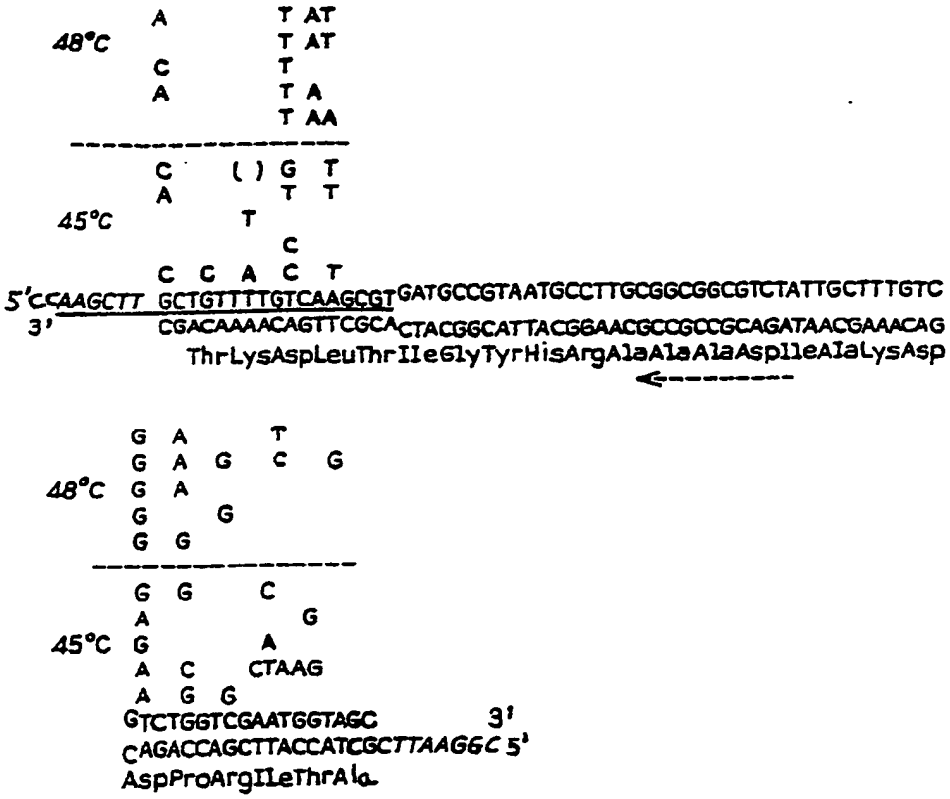


FIG_1

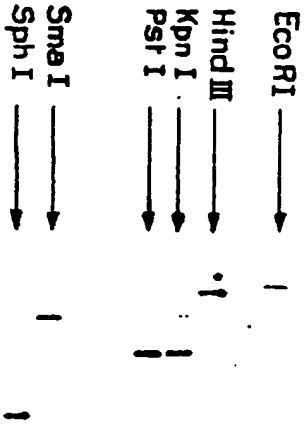
a)


48°C
45°C
5'CCAAGCTT GCTGTTTTGTCAAGCGTGATGCCGTAATGCCTTGCGGCGGCGTCTATTGCTTTGTC
3' CGACAAAACAGTTCGCACTACGGCATTACGGAACGCCGCCGCAGATAACGAAACAG
ThrLysAspLeuThrIleGlyTyrHisArgAlaAlaAlaAspIleAlaLysAsp
48°C
45°C
GTCTGGTCGAATGGTAGC 3'
CAGACCAGCTTACCATCGCTTAAGGC 5'
AspProArgIleThrAla

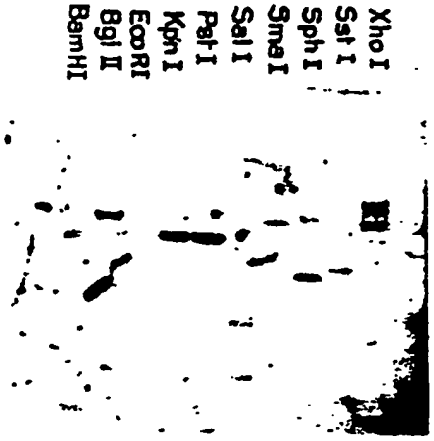
b)


5'
3'
GATGCCGTAATGCCTTGCGGCGGCGTCTATTGCTTTGTCG
SPECIFIC PROBE(Sq918)

FIG_2



Sq 918



Sq 762

Prehybridization ~ 5h à 55°C
hybridization ~ 18h à 55°C

FIG_3

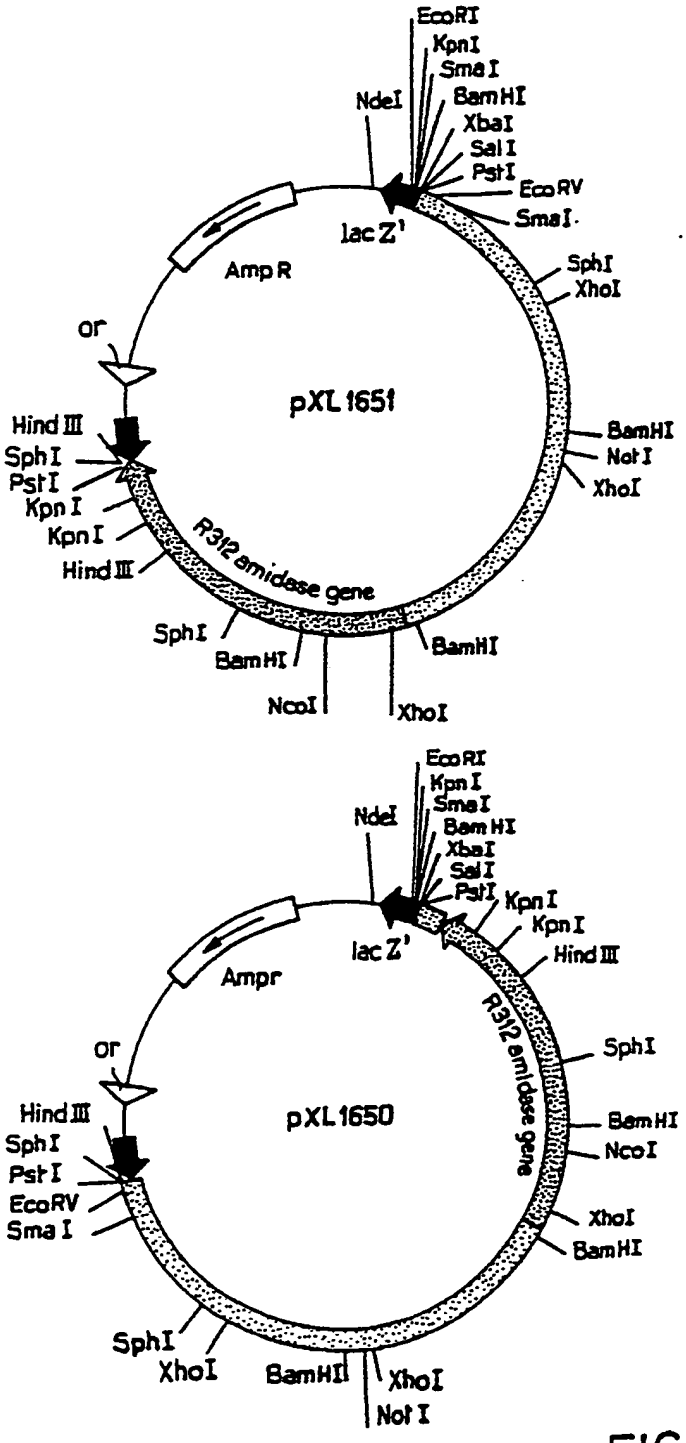

EcoRI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
EcoRV
SmaI
NdeI
lacZ'
AmpR
SphI
XhoI
or
pXL1651
BamHI
NotI
XhoI
HindIII
SphI
PstI
KpnI
KpnI
HindIII
R312 amidase gene
SphI
BamHI
NcoI
XhoI
BamHI
EcoRI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
KpnI
KpnI
HindIII
lacZ'
Ampr
R312 amidase gene
SphI
or
pXL1650
BamHI
NcoI
HindIII
SphI
PstI
EcoRV
SmaI
XhoI
BamHI
SphI
XhoI
BamHI
XhoI
NotI

FIG_4

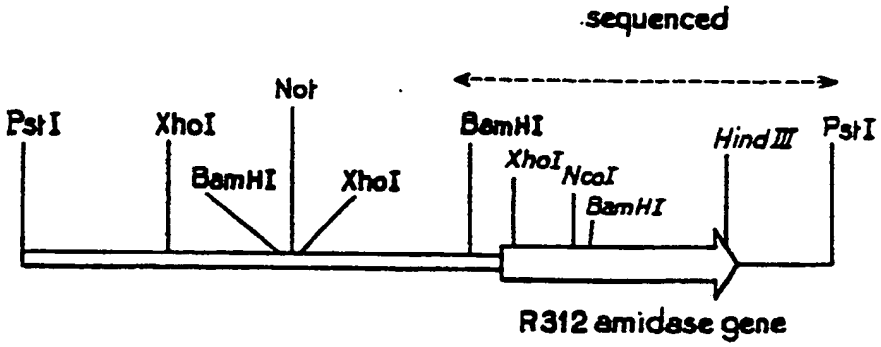

sequenced
Not
PstI
XhoI
BamHI
XhoI
BamHI
XhoI
NcoI
BamHI
HindIII
PstI
R312 amidase gene
5370 pb

FIG_5

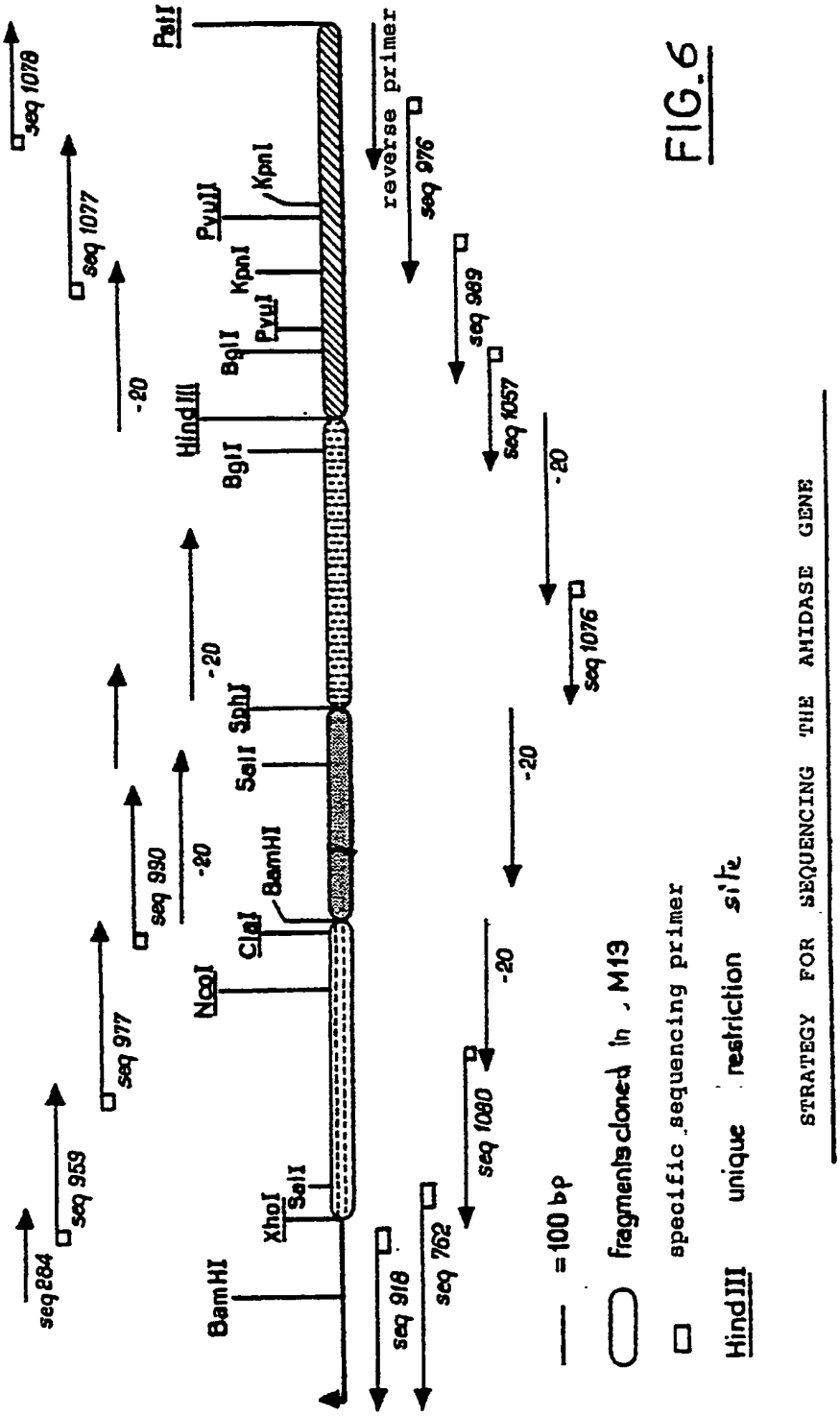


FIG.6

STRATEGY FOR SEQUENCING THE AMIDASE GENE

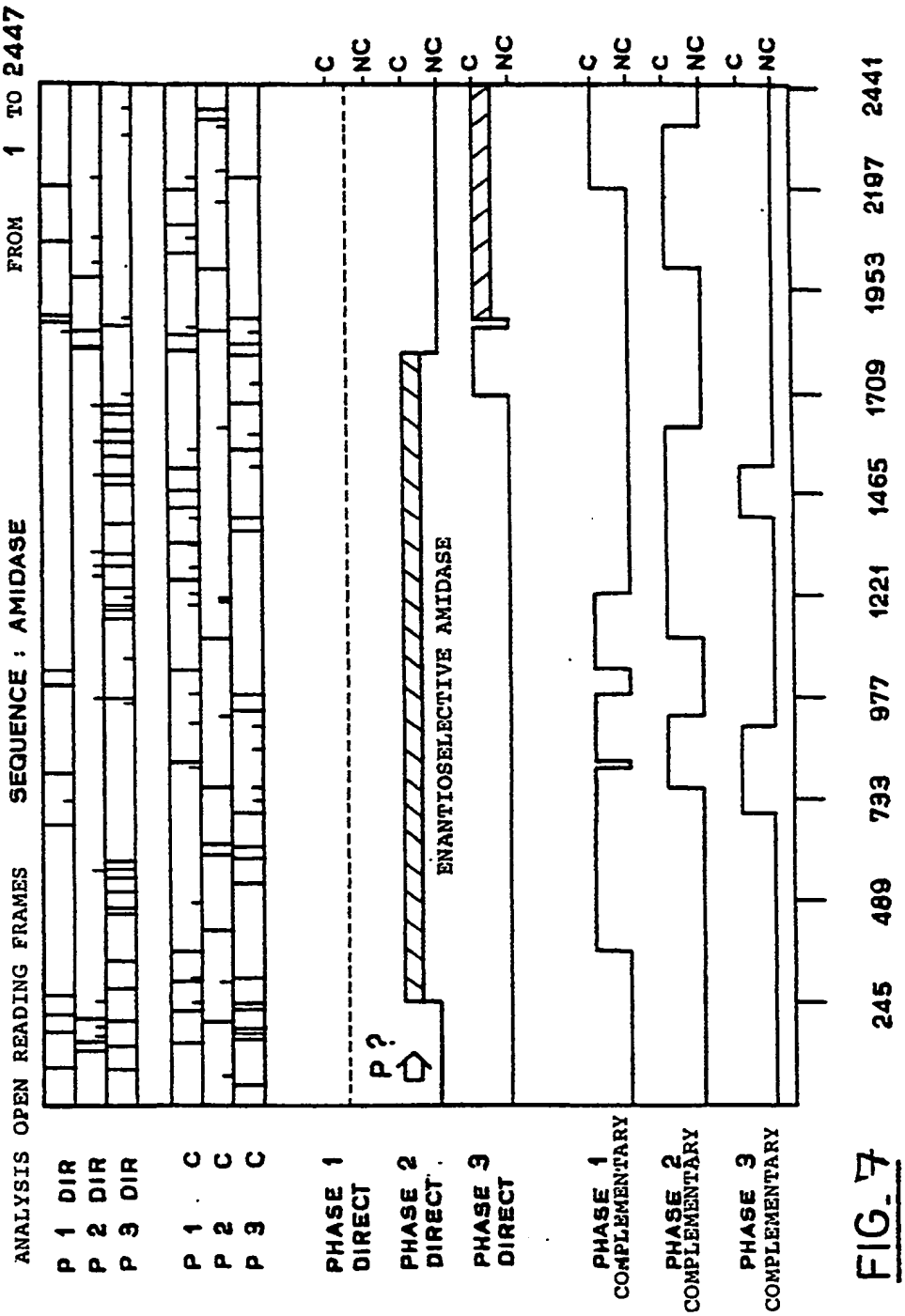

ANALYSIS OPEN READING FRAMES SEQUENCE : AMIDASE FROM 1 TO 2447
P 1 DIR
P 2 DIR
P 3 DIR
P 1 C
P 2 C
P 3 C
PHASE 1 DIRECT
PHASE 2 DIRECT
PHASE 3 DIRECT
PHASE 1 COMPLEMENTARY
PHASE 2 COMPLEMENTARY
PHASE 3 COMPLEMENTARY
P ?
ENANTIOSELECTIVE AMIDASE
C
NC
245 489 733 977 1221 1465 1709 1953 2197 2441

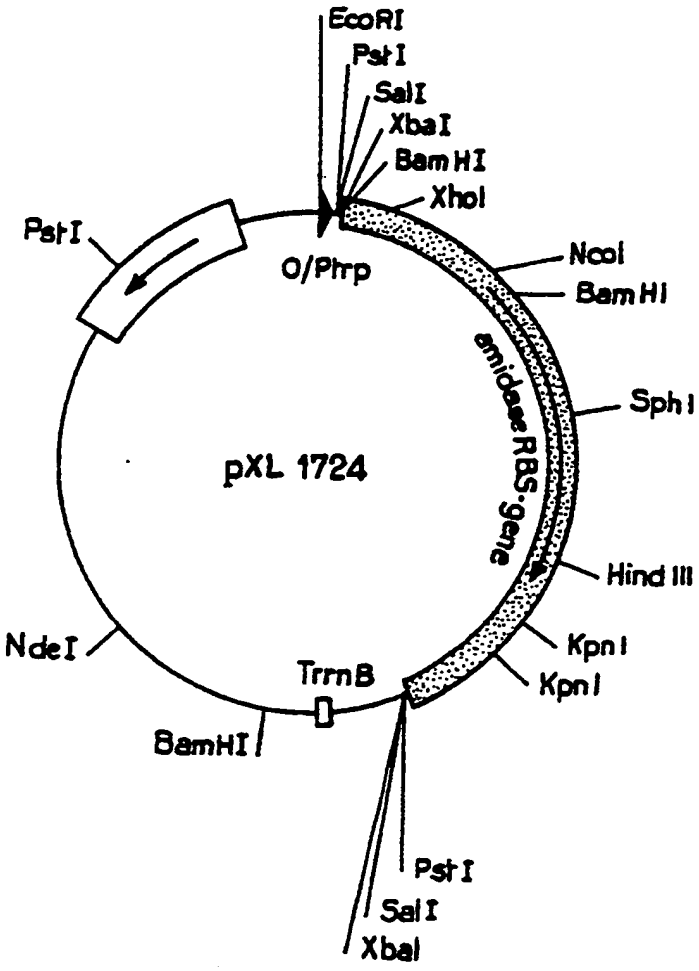
FIG_7

```
cgatccggaaacagtacttcggcagcttgccacgacaccgaaaagctctacgaacaccggtgttccactgca      72

tcggccgattctgatcgctgaatcggcccgtgggcgactgtacccccgctctctctgagcgcacgtaacccg     144
                                                    BamHI
aacttaacgagtcaatatgtcgatacctattgacgcaattatggatccggccctagtctgaaagacaagtga     216
          SD                                amidase ------>
agccgatcacatcaggagcacacttctc ATG GCG ACA ATC CGA CCT GAC GAC AAA GCA ATA     277
                             Met Ala Thr Ile Arg Pro Asp Asp Lys Ala Ile      11

GAC GCC GCC GCA AGG CAT TAC GGC ATC ACT CTC GAC AAA ACA GCC CGG CTC GAG     331
Asp Ala Ala Ala Arg His Tyr Gly Ile Thr Leu Asp Lys Thr Ala Arg Leu Glu      29

TGG CCG GCA CTG ATC GAC GGA GCA CTG GGC TCC TAC GAC GTC GTC GAC CAG TTG     385
Trp Pro Ala Leu Ile Asp Gly Ala Leu Gly Ser Tyr Asp Val Val Asp Gln Leu      47

TAC GCC GAC GAG GCG ACC CCG CCG ACC ACG TCA CGC GAG CAC GCG GTG CCA AGT     439
Tyr Ala Asp Glu Ala Thr Pro Pro Thr Thr Ser Arg Glu His Ala Val Pro Ser      65

GCG AGC GAA AAT CCT TTG AGC GCT TGG TAT GTG ACC ACC AGC ATC CCG CCG ACG     493
Ala Ser Glu Asn Pro Leu Ser Ala Trp Tyr Val Thr Thr Ser Ile Pro Pro Thr      83

TCG GAC GGC GTC CTG ACC GGC CGA CGC GTG GCG ATC AAG GAC AAC GTG ACC GTG     547
Ser Asp Gly Val Leu Thr Gly Arg Arg Val Ala Ile Lys Asp Asn Val Thr Val     101

GCC GGA GTT CCG ATG ATG AAC GGA TCT CGG ACG GTA GAG GGA TTT ACT CCG TCA     601
Ala Gly Val Pro Met Met Asn Gly Ser Arg Thr Val Glu Gly Phe Thr Pro Ser     119

CGC GAC GCG ACT GTG GTC ACT CGA CTA CTG GCG GCC GGT GCA ACC GTC GCG GGC     655
Arg Asp Ala Thr Val Val Thr Arg Leu Leu Ala Ala Gly Ala Thr Val Ala Gly     137

AAA GCT GTG TGT GAG GAC CTG TGT TTC TCC GGT TCG AGC TTC ACA CCG GCA AGC     709
Lys Ala Val Cys Glu Asp Leu Cys Phe Ser Gly Ser Ser Phe Thr Pro Ala Ser     155

GGA CCG GTC CGC AAT CCA TGG GAC CGG CAG CGC GAA GCA GGT GGA TCA TCC GGC     763
Gly Pro Val Arg Asn Pro Trp Asp Arg Gln Arg Glu Ala Gly Gly Ser Ser Gly     173

GGC AGT GCA GCA CTC GTC GCA AAC GGT GAC GTC GAT TTT GCC ATC GGC GGG GAT     817
Gly Ser Ala Ala Leu Val Ala Asn Gly Asp Val Asp Phe Ala Ile Gly Gly Asp     191

CAA GGC GGA TCG ATC CGG ATC CCG GCG GCA TTC TGC GGC GTC GTC GGG CAC AAG     871
Gln Gly Gly Ser Ile Arg Ile Pro Ala Ala Phe Cys Gly Val Val Gly His Lys     209

CCG ACG TTC GGG CTC GTC CCG TAT ACC GGT GCA TTT CCC ATC GAG CGA ACA ATC     925
Pro Thr Phe Gly Leu Val Pro Tyr Thr Gly Ala Phe Pro Ile Glu Arg Thr Ile     227

GAC CAT CTC GGC CCG ATC ACA CGC ACG GTC CAC GAT GCA GCA CTG ATG CTC TCG     979
Asp His Leu Gly Pro Ile Thr Arg Thr Val His Asp Ala Ala Leu Met Leu Ser     245

GTC ATC GCC GGC CGC GAC GGT AAC GAC CCA CGC CAA GCC GAC AGT GTC GAA GCA    1033
Val Ile Ala Gly Arg Asp Gly Asn Asp Pro Arg Gln Ala Asp Ser Val Glu Ala     263
```

FIG. 8-1

```
GGT GAC TAT CTG TCC ACC CTC GAC TCC GAT GTG GAC GGC CTG CGA ATC GGA ATC  1087
Gly Asp Tyr Leu Ser Thr Leu Asp Ser Asp Val Asp Gly Leu Arg Ile Gly Ile   281

GTT CGA GAG GGA TTC GGG CAC GCG GTC TCA CAG CCC GAG GTC GAC GAC GCA GTC  1141
Val Arg Glu Gly Phe Gly His Ala Val Ser Gln Pro Glu Val Asp Asp Ala Val   299

CGC GCA GCG GCA CAC AGT CTG ACC GAA ATC GGT TGC ACG GTA GAG GAA GTA AAC  1195
Arg Ala Ala Ala His Ser Leu Thr Glu Ile Gly Cys Thr Val Glu Glu Val Asn   317
                    SphI
ATC CCG TGG CAT CTG CAT GCT TTC CAC ATC TGG AAC GTG ATC GCC ACG GAC GGT  1249
Ile Pro Trp His Leu His Ala Phe His Ile Trp Asn Val Ile Ala Thr Asp Gly   335

GGT GCC TAC CAG ATG TTG GAC GGC AAC GGA TAC GGC ATG AAC GCC GAA GGT TTG  1303
Gly Ala Tyr Gln Met Leu Asp Gly Asn Gly Tyr Gly Met Asn Ala Glu Gly Leu   353

TAC GAT CCG GAA CTG ATG GCA CAC TTT GCT TCT CGA CGC ATT CAG CAC GCC GAC  1357
Tyr Asp Pro Glu Leu Met Ala His Phe Ala Ser Arg Arg Ile Gln His Ala Asp   371


GCT CTG TCC GAA ACC GTC AAA CTG GTG GCC CTG ACC GGC CAC CAC GGC ATC ACC  1411
Ala Leu Ser Glu Thr Val Lys Leu Val Ala Leu Thr Gly His His Gly Ile Thr   389

ACC CTC GGC GGC GCG AGC TAC GGC AAA GCC CGG AAC CTC GTA CCG CTT GCC CGC  1465
Thr Leu Gly Gly Ala Ser Tyr Gly Lys Ala Arg Asn Leu Val Pro Leu Ala Arg   407

GCC GCC TAC GAC ACT GCC TTG AGA CAA TTC GAC GTC CTG GTG ATG CCA ACG CTG  1519
Ala Ala Tyr Asp Thr Ala Leu Arg Gln Phe Asp Val Leu Val Met Pro Thr Leu   425

CCC TAC GTC GCA TCC GAA TTG CCG GCG AAG GAC GTA GAT CGT GCA ACC TTC ATC  1573
Pro Tyr Val Ala Ser Glu Leu Pro Ala Lys Asp Val Asp Arg Ala Thr Phe Ile   443

ACC AAG GCT CTC GGG ATG ATC GCC AAC ACG GCA CCA TTC GAC GTG ACC GGA CAT  1627
Thr Lys Ala Leu Gly Met Ile Ala Asn Thr Ala Pro Phe Asp Val Thr Gly His   461

CCG TCC CTG TCC GTT CCG GCC GGC CTG GTG AAC GGG CTT CCG GTC GGA ATG ATG  1681
Pro Ser Leu Ser Val Pro Ala Gly Leu Val Asn Gly Leu Pro Val Gly Met Met   479

ATC ACC GGC AGA CAC TTC GAC GAT GCG ACA GTC CTT CGT GTC GGA CGC GCA TTC  1735
Ile Thr Gly Arg His Phe Asp Asp Ala Thr Val Leu Arg Val Gly Arg Ala Phe   497
      HindIII
GAA AAG CTT CGC GGC GCG TTT CCG ACG CCG GCC GAA CGC GCC TCC AAC TCT GCA  1789
Glu Lys Leu Arg Gly Ala Phe Pro Thr Pro Ala Glu Arg Ala Ser Asn Ser Ala   515

CCA CAA CTC AGC CCC GCC  tagtcctgacgcactgtcagacaacaaattccaccgattcacacatg 1854
Pro Gln Leu Ser Pro Ala                                                   521

atcagcccacataagaaaaggtgaa
```

FIG_8·2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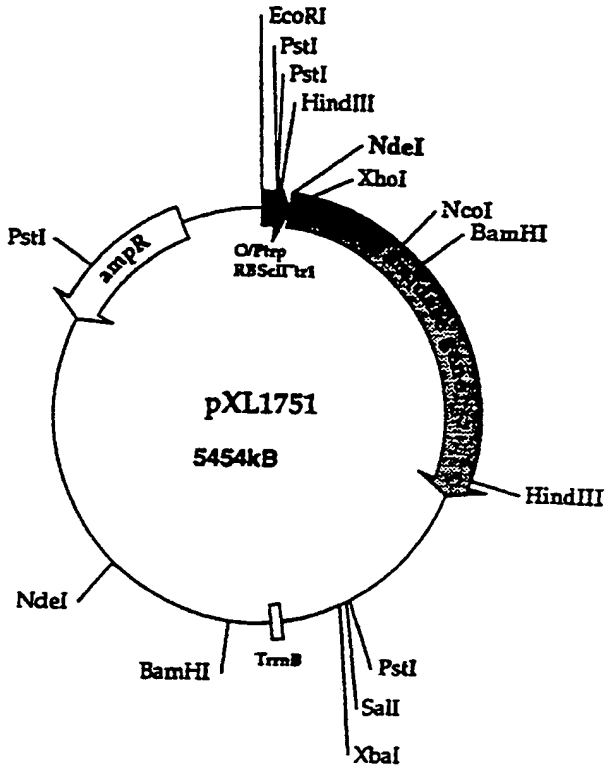
SalI
XbaI
BamHI
XhoI
NcoI
BamHI
SphI
Hind III
KpnI
KpnI
PstI
SalI
XbaI
BamHI
NdeI
PstI
O/Ptrp
TrrnB
pXL 1724
amidase RBS gene

FIG_9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XhoI
NcoI
BamHI
PstI
ampR
O/Ptrp
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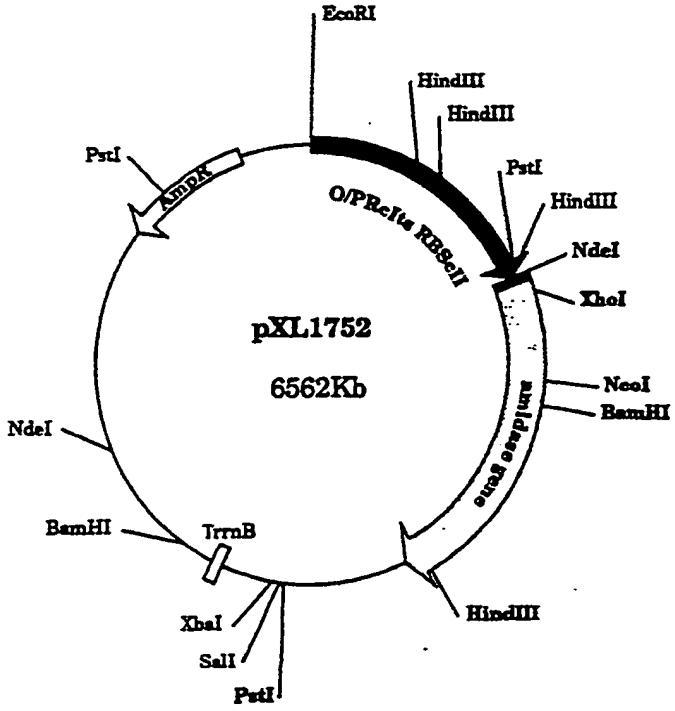
pXL1751
5454kB
HindIII
NdeI
BamHI
TrrnB
PstI
SalI
XbaI

FIG_10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PstI
HindIII
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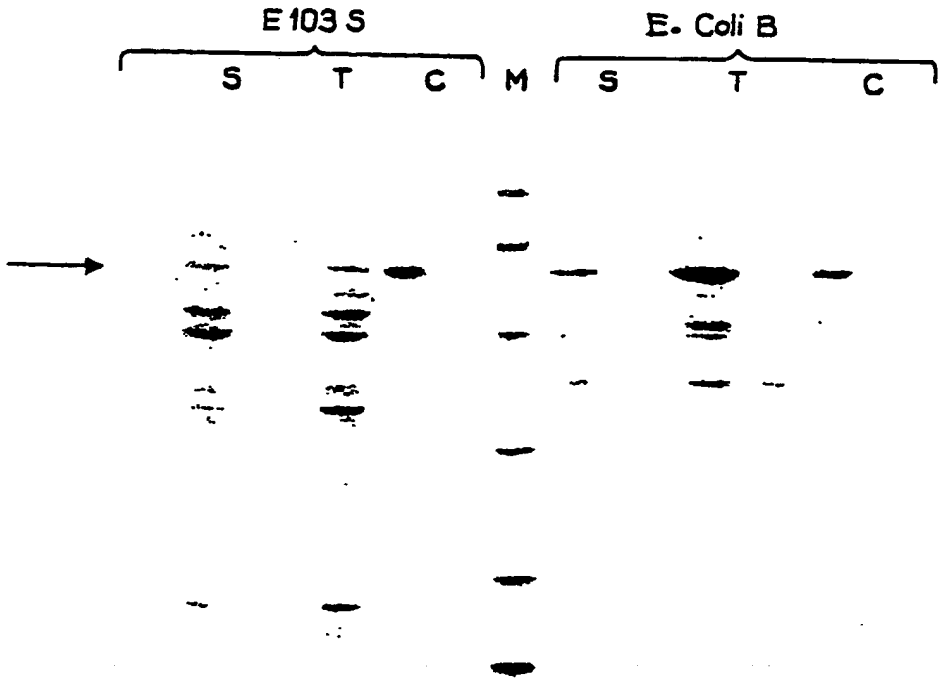
XhoI
NcoI
BamHI
pXL1752
6562Kb
NdeI
BamHI
TrrnB
XbaI
SalI
PstI
HindIII

FIG_11



1029	1752	1029	1752	1029	1751	906	1751	906	1751	906	plasmid
42°	42°	42°	42°	42°	+	-	+	-	+	-	trp

S : Supernatants

T : Total

C : Pellet

M : Marqueur

pxL 1029 : IL1β under control of P_{RcIts}

pxL 906 : " " " Ptrp

FIG_12

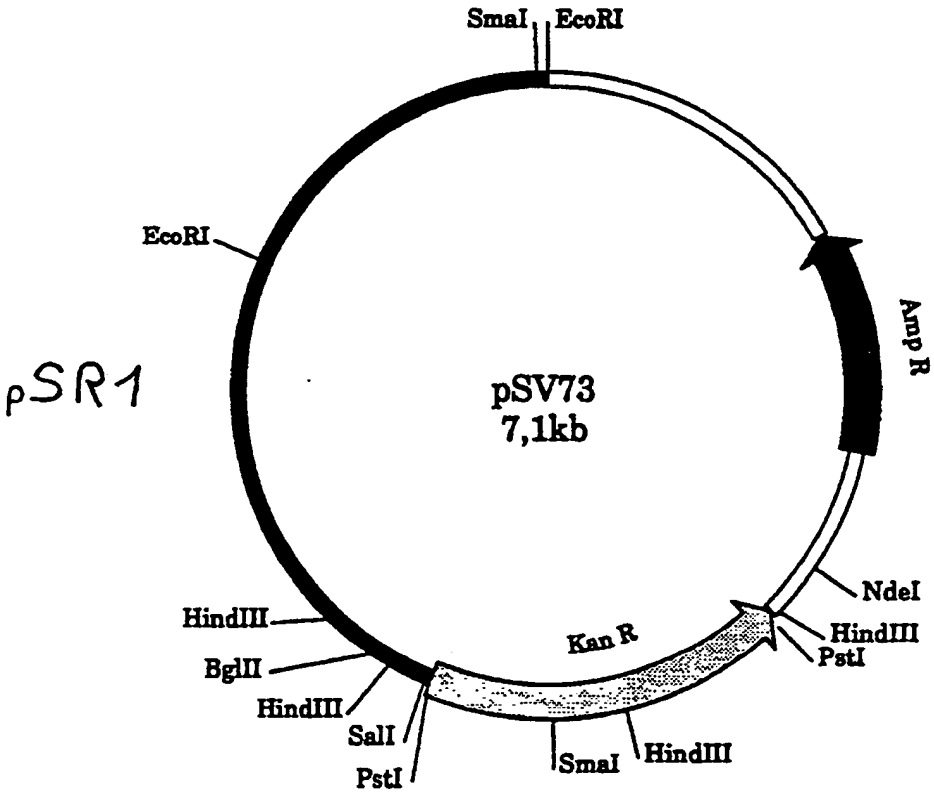
BamHI, SalI, PstI (polylinker)

```
ctgcagaacggaactaagatggctcgaaccttcaccaaagacggacttgaacacagcctcgcacttgcgcgt   72

ttggagctcccggacgagcgttacgagacggtgacagcggctgccgagttggtcctcggactcgctgaggct  144

                                                                    NdeI
                                                                   cat
ctggatgctgtcccgctggccgagactccgatggcagccgccttcgatgcgcggtgggagtgacg ATG      212
                                                                   Met       1

GGC TTG CAT GAA CTG ACG CTC GCG CAA GTC GCT GCG AAG ATC GAG AAC AAA GAA     266
Gly Leu His Glu Leu Thr Leu Ala Gln Val Ala Ala Lys Ile Glu Asn Lys Glu    18

CTT TCC CCG GTC GAG CTC CTC GAT GTG ATC CTG GCG CGC GTC GCG GAG ATC GAA    320
Leu Ser Pro Val Glu Leu Leu Asp Val Ile Leu Ala Arg Val Ala Glu Ile Glu    36

CCG AAG ATC TCC GCC TTC GTC ACG ATC ACC GCC GAT TCC GCT CGG AAG GCG GCC    374
Pro Lys Ile Ser Ala Phe Val Thr Ile Thr Ala Asp Ser Ala Arg Lys Ala Ala    55

CGG CTC GCA GCC GAC GAG ATC GCA GGT GGG CAC TAT CGC GGT CCG CTG CAC GGA    428
Arg Leu Ala Ala Asp Glu Ile Ala Gly Gly His Tyr Arg Gly Pro Leu His Gly    73

GTT CCG ATT GGC CTC AAG GAT CTG TTC GAA GTG GCA GGC GTC CCG AAT ACC GCG    482
Val Pro Ile Gly Leu Lys Asp Leu Phe Glu Val Ala Gly Val Pro Asn Thr Ala    91

AGT TCG CGG GTC CGA GCT GAC TAC ATC CCC TCA TCG GAT GGG GCC GCG GTC GAG    536
Ser Ser Arg Val Arg Ala Asp Tyr Ile Pro Ser Ser Asp Gly Ala Ala Val Glu   109

AAG CTC ACC GCC GGT GGA GCG GTC ATG ATC GGC AAG ACG CAC ACT CAC GAA TTC    590
Lys Leu Thr Ala Gly Gly Ala Val Met Ile Gly Lys Thr His Thr His Glu Phe   127

GCC TAC GGT GCG ATC ACA CCG ACC ACC CGT AAT CCA TGG GAC CCC ACC CGG ACA    644
Ala Tyr Gly Ala Ile Thr Pro Thr Thr Arg Asn Pro Trp Asp Pro Thr Arg Thr   145

CCC GGC GGT TCC AGC GGT GGG ACG GCA GCA GCT CTC GCG GCA GGC CTC ATC TTC    698
Pro Gly Gly Ser Ser Gly Gly Thr Ala Ala Ala Leu Ala Ala Gly Leu Ile Phe   163

GCC GGT ATG GGT ACC GAT ACC GGG GGG TCC ATT CGG ATA CCA GCC GCC GTC TGC    752
Ala Gly Met Gly Thr Asp Thr Gly Gly Ser Ile Arg Ile Pro Ala Ala Val Cys   181

GGG ACG GTA GGT CTC AAA CCC ACA TAT GGT CGC GTT TCG CGT CGT GGA GTG ACC    806
Gly Thr Val Gly Leu Lys Pro Thr Tyr Gly Arg Val Ser Arg Arg Gly Val Thr   199

TCC TTG TCA TGG TCT CTG GAC CAC GCG GGA CCG CTG GCC CGG ACC GTG GAA GAC    860
Ser Leu Ser Trp Ser Leu Asp His Ala Gly Pro Leu Ala Arg Thr Val Glu Asp   217

GCT GCC ATC ATG CTG AAC CAG ATC GCT GGC TAT GAC CGG GCT GAT CCT GCG ACG    914
Ala Ala Ile Met Leu Asn Gln Ile Ala Gly Tyr Asp Arg Ala Asp Pro Ala Thr   235

GTA GAT GTG CCC GTT CCC GAC TAC GCG GCG GCG CTG ACC GGA GAC GTC CGA GGG    968
Val Asp Val Pro Val Pro Asp Tyr Ala Ala Ala Leu Thr Gly Asp Val Arg Gly   253

CTG CGG ATT GGT GTG CCG ACC AAT TTC TAC ACC GAC AAC GTC CAT CCC GAG GTT   1022
Leu Arg Ile Gly Val Pro Thr Asn Phe Tyr Thr Asp Asn Val His Pro Glu Val   271

GCC GCA GCG GCC GAC GCT GCG GTG GCG CAA CTG GCC CAT TTG GGT GCG GTG GTC   1076
Ala Ala Ala Ala Asp Ala Ala Val Ala Gln Leu Ala His Leu Gly Ala Val Val   289

CGC GAA GTG AAG ATC CCG ATG GCA GAG GTC ATC GTG CCC ACC GAG TGG AGC TTG   1130
Arg Glu Val Lys Ile Pro Met Ala Glu Val Ile Val Pro Thr Glu Trp Ser Leu   307

CTC GTC CCG GAG GCG TCG GCC TAC CAC CAG CAG ATG CTG CGC GAG CGC GCA GAT   1184
Leu Val Pro Glu Ala Ser Ala Tyr His Gln Gln Met Leu Arg Glu Arg Ala Asp   325

CAC TAC ACC GAC GAG ACG AGA ACC TTC CTG GAA GCC GGC GAA CTC GTT CCG GCG   1238
His Tyr Thr Asp Glu Thr Arg Thr Phe Leu Glu Ala Gly Glu Leu Val Pro Ala   343
```

FIG 13-1

```
ACC GAC TAC ATC AAG GCG CTG CGG GTG CGC ACC CTC ATC CAG GCA GCC TTC CGG  1292
Thr Asp Tyr Ile Lys Ala Leu Arg Val Arg Thr Leu Ile Gln Ala Ala Phe Arg   361

GAA CTG TTC CAG GAC ATC GAT GTC CTG ATC GCA CCC ACG GTC AGC TCT CCG GCT  1346
Glu Leu Phe Gln Asp Ile Asp Val Leu Ile Ala Pro Thr Val Ser Ser Pro Ala   379

CTG CCG CTC GAT GAC CTG GAA GTC ACT TGG CCC GAT GGC ACA TCC GAA GGC GGC  1400
Leu Pro Leu Asp Asp Leu Glu Val Thr Trp Pro Asp Gly Thr Ser Glu Gly Gly   397

ACC ATC ACC TAT GTC CGT CTC AGC GCC CCC GGC AAC GTC ACC GGA CTT CCA GCG  1454
Thr Ile Thr Tyr Val Arg Leu Ser Ala Pro Gly Asn Val Thr Gly Leu Pro Ala   415

CTG TCG GTC CCC TCC GGC TTC ACC GAG CAA GGC CTT CCC ACC GGT ATC CAG ATC  1508
Leu Ser Val Pro Ser Gly Phe Thr Glu Gln Gly Leu Pro Thr Gly Ile Gln Ile   433

ATC GGC CGT CCC TTC GAC GAG GAG ACC GTC CTC AAC GTC GGT CAC GCC TAC GAA  1562
Ile Gly Arg Pro Phe Asp Glu Glu Thr Val Leu Asn Val Gly His Ala Tyr Glu   451

GGC TGC ACG GAC TGG CCG CGA CTG GCG CCG CTT TGA  actactgacccccattggagaaa 1621
Gly Cys Thr Asp Trp Pro Arg Leu Ala Pro Leu                               463

                                                                  SalI
accgaaggagagaacgatgaatggagtgttcgatttgggtgggaccgacggcatcggcccggtcgaccctcc 1693


cgctgaagaaccggtgttccgcgcggactgggagaaagcagccttcaccatgttctcggcgctattccgtgc 1765

                                                  BamHI
cggctggttcggcatcgacgaattccgtcacggtgtcgaaaagatggatcc
```

FIG 13-2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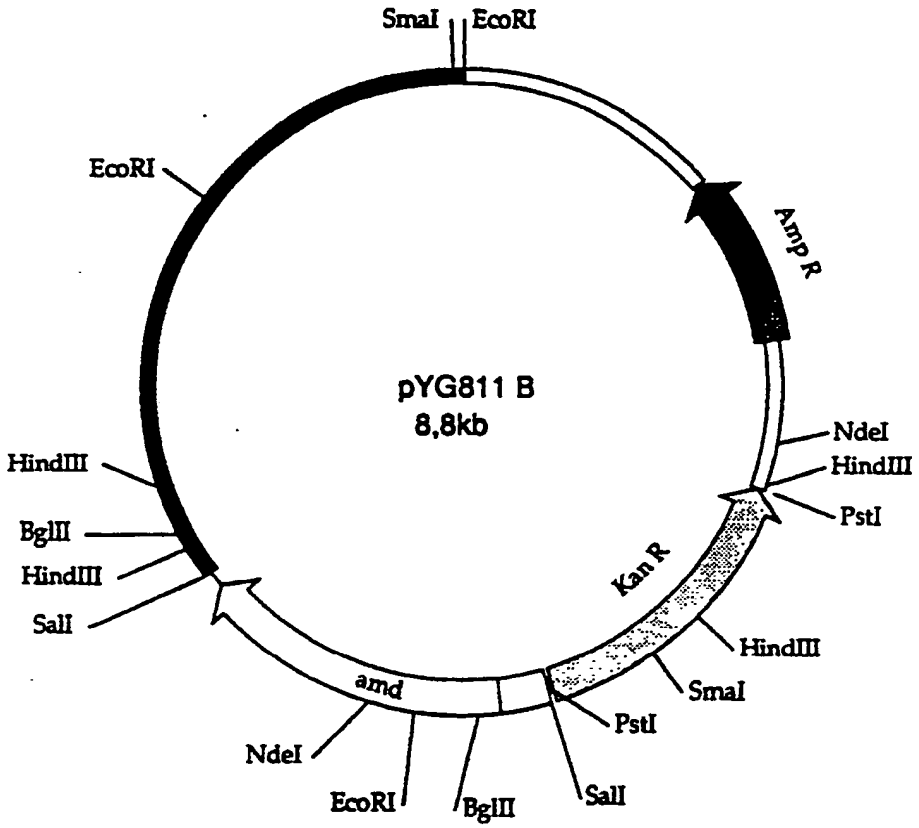
PstI
SmaI
HindIII

pSR1

FIG 14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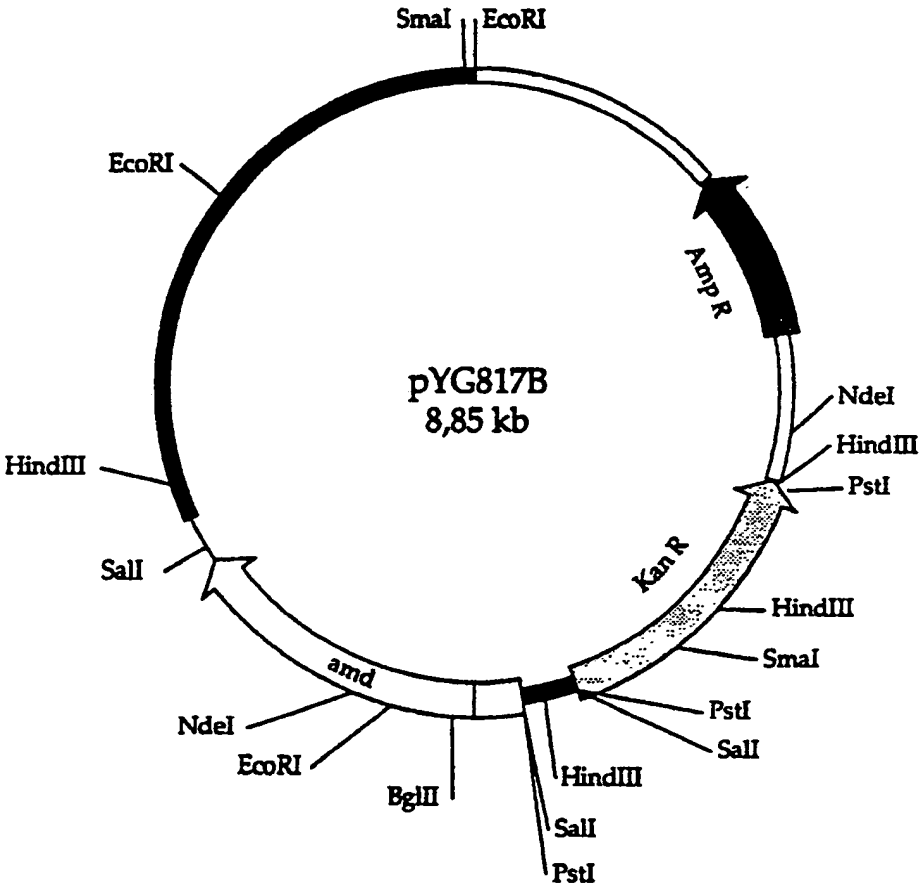
SalI

FIG 15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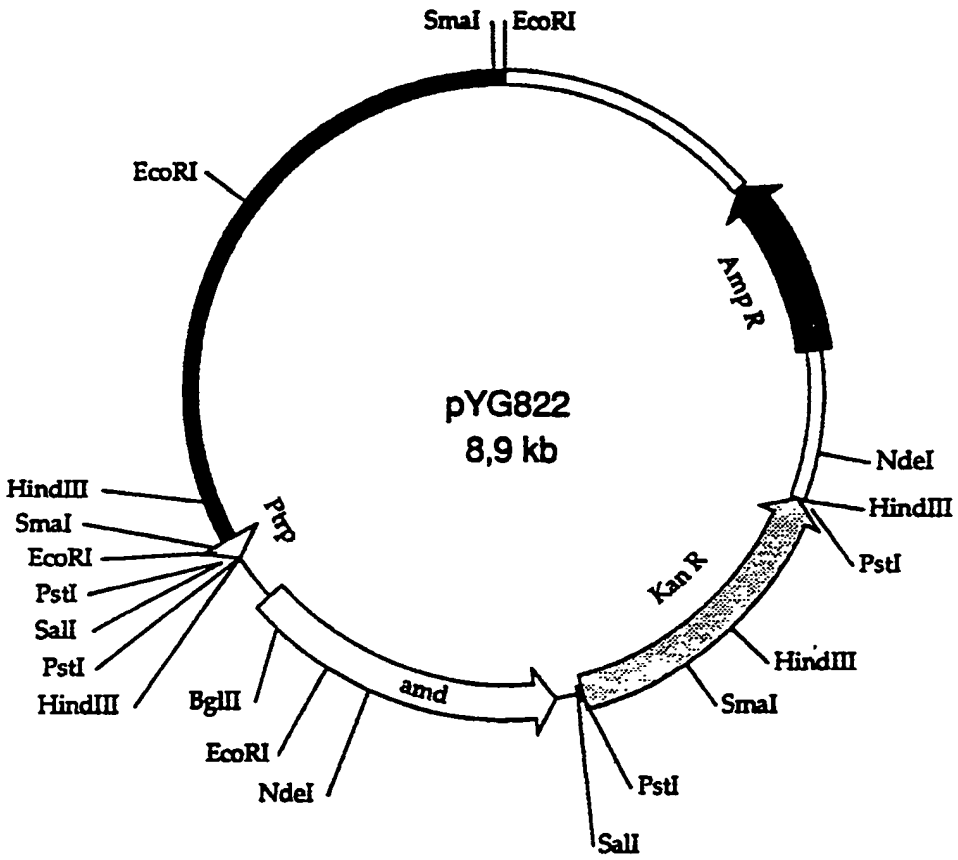
SalI
PstI

FIG 16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SalI
PstI
HindIII
PtrP
BglII
EcoRI
NdeI
amd
Kan R
PstI
SalI

17

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 40 3232

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-326482 (RHONE-POULENC SANTE) * pages 2 - 3 *	24-29	C12N15/55 C12P7/40 C12P41/00
A,D	EP-A-330529 (RHONE-POULENC SANTE) * the whole document *	24-29	C12N9/80 //(C12N1/21; C12R1:19,1:15, 1:13,1:01)
A	GB-A-2218985 (SHELL INTERNATIONALE RESEARCH MAATSCHAPPIJ_B.V.) * the whole document *	24-29	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C12N
C12P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	11 MARCH 1991	ANDRES S.M.

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